In vitro elimination of *Black raspberry necrosis virus* from black raspberry (*Rubus occidentalis*) – Short communication

E.J. Cheong, A.R. Jeon, J.W. Kang, R. Mock, G. Kinard, R. Li

National Germplasm Resources Laboratory, Agricultural Research Service, United States Department of Agriculture, Beltsville, USA

Abstract

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Black raspberry necrosis virus (BRNV) is one of the most important viral pathogens of *Rubus* spp. In this study, a procedure combining *in vitro* culture and heat therapy on axillary buds of *Rubus occidentalis* was developed to eliminate BRNV from infected plants. Axillary buds were grown aseptically at 4-h alternating periods of 29°C and 38°C with 14-h day length for 1–5 weeks. Shoots induced after thermotherapy were tested for the viruses by RT-PCR. BRNV was not detected in any shoots indicating that BRNV was heat labile. BRNV-free shoots were rooted *in vitro* and acclimatized in greenhouse. Regenerated plants remained virus-free after 2-month acclimatization, 3-month dormancy and 5-month growth periods.

Keywords: BRNV; *Rubus occidentalis*; axillary bud culture; thermotherapy

Raspberries are very important small fruit crops in the genus *Rubus* of the family Rosaceae. Their berry fruits are a rich source of soluble fibre, vitamins, minerals and natural antioxidants. In recent years, consumer demand for these fruits has led to an increase in their worldwide production. Raspberries have a long history of cultivation and are produced worldwide. Red raspberries (*R. idaeus*) are currently the most widely grown worldwide, with the U.S. as the third largest producer. Black raspberries (*R. occidentalis*) are more popular in the U.S. Black raspberries, which are native to North America, are the third most popular berry in the U.S. behind strawberry and blueberry (Pollack, Perez 2006). More than 40 viruses and virus-like pathogens have been reported to infect *Rubus* spp., causing serious yield loss and decline of *Rubus* plantings worldwide (McGavin et al. 2010). The pathogens are easily spread because these crops are usually propagated and distributed as vegetative cuttings. *Black raspberry necrosis virus* (BRNV) is the most common aphid-borne virus of raspberry associated with mosaic disease throughout the world (Stace-Smith 1955; Schliephake 2010). It is a member of the genus *Sadwavirus* in the family Secoviridae. The host range of the virus is restricted to *Rubus* species where it causes chlorosis along the leaf veins (Jones et al. 2002; Martin 2002; Halgren et al. 2007).

The distribution of healthy plant material is an important measure to prevent the spread of destructive viral pathogens that can accompany the movement of plant germplasm. Elimination methods such as meristem culture, thermotherapy and chemotherapy, either alone or in combination, are usually used to obtain virus-free plants from in-

fected mother plants (Mellor, Stace-Smith 1978; Baumann 1982; Karesova et al. 2002; Hosokawa et al. 2004; Sharma et al. 2008; Cheong et al. 2012). We report here a simple and effective procedure for the elimination of BRNV from black raspberry by *in vitro* axillary bud culture and thermotherapy.

MATERIALS AND METHODS

Plant materials and *in vitro* culture. Stem cuttings from BRNV-infected black raspberry plants were obtained from Dr. Robert Martin (USDA-ARS, Corvallis, USA). The plants were established and maintained in an insect-proof screen house. Presence of the virus in the mother plants was confirmed by RT-PCR as described below. Actively growing shoots were collected during the growing season (late June through August) from infected plants. The cuttings were surface-sterilized with 1% NaOCl for 10 min, 70% ethanol for 5 min and rinsed three times with double sterilized water. Explants, each containing a single axillary bud, were placed on corresponding shoot initiation medium (8 ml) in glass tubes of 150 × 25 mm. Both shoot and root induction media were based on Murashige and Skoog (1962) basal salts with vitamins (Table 1). Cultures were maintained at 22°C with 16-h photoperiod under cool white fluorescent lights (40–50 μmol/m^2·s).

Thermotherapy. Heat treatment was performed on *in vitro* axillary bud cultures in a growth chamber (Model I-36VL; Percival, Perry, USA) where the temperature was alternated every 4 h between 29°C and 38°C to avoid continuous extreme condition that results plant death. Explants were cultivated for 5 weeks under this high temperature regime in the initial experiment. In a second experiment, various periods (1 to 5 weeks) under the high temperature regime were investigated. Explants were removed from the heat treatment every week and transferred to regular temperature (22°C) for recovery. After 4 weeks of the recovery culture, samples of each treatment were collected for virus detection.

Root induction and acclimatization in greenhouse. After the recovery culture, RT-PCR-negative plantlets (ca. 3 cm or more in length) were transferred onto root-induction medium. The plantlets were maintained under low light for one week and then transferred to regular light conditions. Within 4–8 weeks, rooted plantlets of 3 cm or higher were transferred to soil (Premier™ Pro-Mix HP, Quakertown, USA) covered with clear plastic cups and maintained in a growth chamber at 22°C with 16-h photoperiod. Four to six weeks later, acclimatized plants were transferred to a greenhouse and maintained there for 2 months. To simulate dormancy, the plants were pruned and maintained in a growth chamber with the following treatment: 15°C day and 10°C night with a 10-h photoperiod for 16 days, 10°C day and 5°C night with a 9-h photoperiod for 14 days, 5°C day and 0°C night with a 8-h photoperiod for 35 days and 5°C day and 2°C night with an 8-h photoperiod for 20 days. After dormancy, plants were transferred to an ambient greenhouse (ca. 25°C day and 21°C night) for initiation of new growth.

Virus detection. Leaf tissues were collected from: regenerated shoots 5–9 weeks after in *vitro* culture; plantlets after the recovery growth; and plants after the 3-month dormancy period. Total nucleic acids were extracted using a CTAB method (Li et al. 2008) and used as templates in RT-PCR for virus detection. The RT-PCR was performed using the SuperScript™ III One-Step RT-PCR System (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. BRNV was detected in RT-PCR

Table 1. Components of media[1] in each stage of *in vitro* culture for *Rubus* species

	Shoot initiation	Root induction
6-Benzylaminopurine (mg/l)	0.25	–
Sequestrene (mg/l)	50	50
Sucrose (g/l)	30	30
pH	5.7	5.7
Agar (g/l)	3	3
Gelrite (g/l)	1.25	1.25

[1]MS basal salts and vitamins as basal medium

after the recovery culture by primers BRNV 1F (5'-ATGCTGAGCCACTTGTGA-3') and BRNV1R (5'-ATCTGGTGTGTTCCGCAT-3') (Halgren et al. 2007).

Statistical Analysis. In the first experiment, 104 explants in total (24, 40 and 40 per replication) were tested. In the second experiment, 18–20 explants were cultivated per treatment and replicated twice. Regenerated shoots in each treatment and survived plants after acclimatization and dormancy were tested by RT-PCR for the presence of the virus. Regeneration rate was determined four weeks after the culture was initiated. Explants were considered regenerated if new leaves and shoot grew and persisted on fresh media. The virus-free rate was the number of virus-free plants/number of elongated explants × 100. The statistical significance of all criteria was evaluated using ANOVA at a level of $P = 0.05$: regeneration and virus-free rate.

RESULTS AND DISCUSSION

We described the effect of heat treatment on BRNV and developed a protocol combining *in vitro* culture and heat treatment on axillary buds from infected black raspberry (*Rubus occidentalis*). In the first experiment, all (100%) of the regenerated shoots under 5 week-heat treatment were negative for BRNV, which draw the second experiment investigating the effect of the thermotherapy duration (1–5 weeks). Heat-treated explants from infected black raspberry produced shorter shoots and smaller leaves when compared with those of control explants grown at 22°C (Fig. 1). Regeneration rates (ratio of the number of elongated shoots to the total number of cultured explants) of heat treated explants were slightly lower than control explants. However, both regeneration rates and virus elimination rates among the length of heat treatment were not significantly different (Table 2). Only one week of the thermotherapy resulted in 100% BRNV elimination. While limited virus-free rates after the thermotherapy were obtained for viruses such as *Rubus yellow net virus* (RYNV) (Mellor, Stace-Smith 1980), *Raspberry vein chlorosis virus* (Baumann 1982) and *Raspberry bushy dwarf virus* (RBDV) (Lankes 1995; Karesova et al. 2002; Wang et al. 2008), the full success we obtained in BRNV eradication may be explained by the fact that BRNV was heat labile (Cadman 1961; Stace-Smith, Mellor 1978). The result showed that heat treatment greatly affected the replication of BRNV. Growing host plants at elevated temperature can inhibit virus replication and movement. It was recently shown that viral RNA of RBDV, a virus that enters meristem tissues, was disorganized in leaves and shoot tips in plants growing at high temperatures (38°C) (Wang et al. 2008). This was attributed to the enhancement of RNA silencing, a

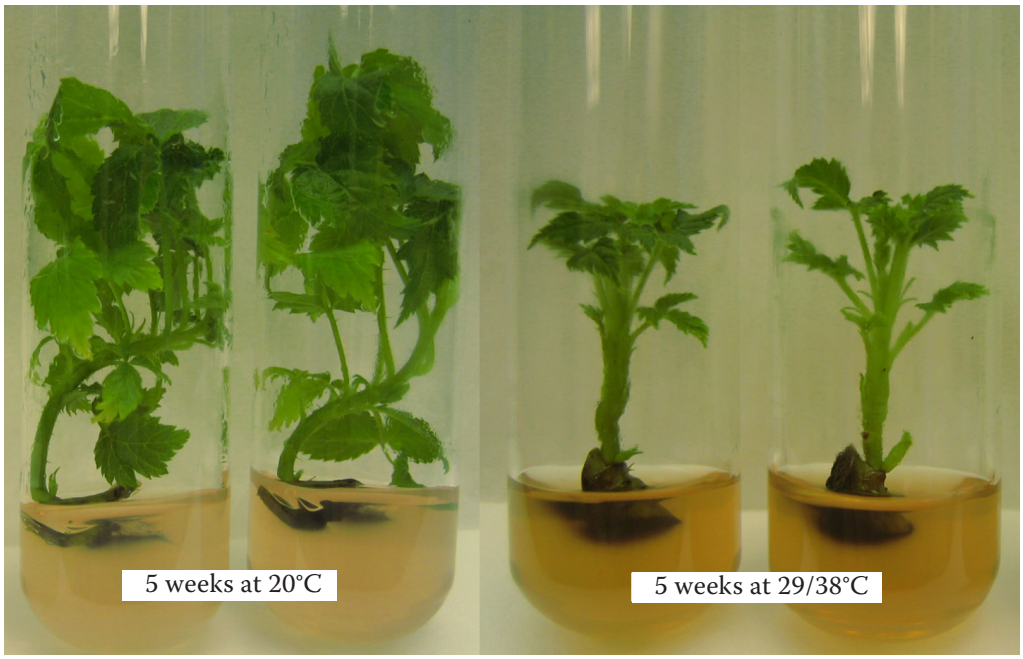

Fig. 1. Comparison of *in vitro* shoots of *R. occidentalis* between control plants at 22°C and heat-treated (29/38°C) after 5 weeks in culture

Table 2. Shoot regeneration and virus elimination rates[1] for BRNV

Weeks in heat[2]	Regeneration (%)[3]	Elimination (%)
0	36/37 (97.3 ± 3.7)[a]	2/36 (5.5)
1	35/40 (87.5 ± 3.5)[b]	35/35 (100)
2	34/40 (85.0 ± 0.0)[b]	34/34 (100)
3	32/40 (85.0 ± 0.0)[b]	32/32 (100)
4	35/40 (87.5 ± 3.5)[b]	35/35 (100)
5	36/40 (90.0 ± 7.1)[b]	36/36 (100)

[1]after heat treatment, explants were placed at 22°C for 4 weeks before data collection and virus detection; [2]number of weeks in heat treatment; [3]mean ± standard deviation; [a, b]at $P = 0.05$, ANOVA indicated that there was no statistically significant difference (same letters) in survival rate and virus-free rates between the durations of heat treatments except the control

cellular defence mechanism of plants acting against viral RNA, in higher temperatures (Chellappan et al. 2005). Although thermotherapy was successfully used along with *in vitro* shoot tip culture to produce virus-free plants (Karesova et al. 2002; Wang et al. 2006; Sharma et al. 2008; Panattoni, Triolo 2010; Cheong et al. 2012), higher efficacy of virus elimination was achieved in combination with *in vitro* meristem tip culture for most viruses. One-hundred percentage elimination of BRNV by thermotherapy and shoot tip culture is unusual, suggesting the virus is completely inhibited by heat stress. Further study of this system will be useful to explore the effect of elevated temperature on plant pathogen interactions.

In vitro thermotherapy of explants without meristem tip culture was sufficient to eliminate this virus. In addition, the short period of thermotherapy for elimination of BRNV will be especially useful to produce virus-free germplasm or mother plants for quarantine and certification programs. This will in turn facilitate the safe exchange and distribution of *Rubus* spp. and help broaden the gene pool from which breeders can develop new and improved varieties.

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Corresponding author:

Dr. Ruhui Li, United States Department of Agriculture, Agricultural Research Service, National Germplasm Resources Laboratory, 10300 Baltimore Av., Beltsville, MD 20705, USA
phone: + 1 301 504 7653, e-mail: Ruhui.Li@ars.usda.gov